CARDIOPROTECTIVE EFFECTS OF INGLIFORIB, A NOVEL GLYCOGEN PHOSPHORYLASE INHIBITOR

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Running title: Glycogen phosphorylase inhibitor reduces ischemic injury

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Tracey, W. Ross, Judith L. Treadway, William P. Magee, Jill C. Sutt, R. Kirk McPherson, Carolyn B. Levy, Donald E. Wilder, Li J. Yu, Yue Chen, Ravi M. Shanker, Alison K. Mutchler, Andrew H. Smith, David M. Flynn and Delvin R. Knight. Cardioprotective effects of ingliforib, a novel glycogen phosphorylase inhibitor. —— Interventions such as glycogen depletion that limit myocardial anaerobic glycolysis and the associated proton production, can reduce myocardial ischemic injury; thus, it follows that inhibition of glycogenolysis should also be cardioprotective. Therefore, we examined whether a novel glycogen phosphorylase inhibitor, ingliforib (CP-368,296), could reduce infarct size in both in vitro and in vivo rabbit models of ischemia-reperfusion injury (30 min regional ischemia, followed by 120 min reperfusion). In Langendorff hearts, constant perfusion of ingliforib, started 30 min before regional ischemia, elicited a concentration-dependent reduction in infarct size; infarct size was reduced by 69% with 10 µM ingliforib. No significant drug-induced changes were observed in either cardiac function (heart rate, left ventricular developed pressure) or coronary flow. In open-chest, anesthetized rabbits, a dose of ingliforib (15 mg/kg loading dose; 23 mg/kg/hr infusion) selected to achieve a free plasma concentration equivalent to an estimated EC₅₀ in the isolated hearts (1.2 µM, 0.55 µg/ml) significantly reduced infarct size by 50%, and reduced plasma glucose and lactate concentrations. Furthermore, myocardial glycogen phosphorylase α and total glycogen phosphorylase activity were reduced by 65% and 40%, respectively, and glycogen stores preserved, in ingliforib-treated hearts. No significant change was observed in mean arterial pressure or rate pressure product in the ingliforib group, although heart rate was modestly decreased post-ischemia. In conclusion, glycogen phosphorylase inhibition with ingliforib markedly reduces myocardial ischemic injury in vitro and in vivo; this may represent a viable approach
for both achieving clinical cardioprotection, and treating diabetic patients at increased risk of cardiovascular disease.

**Keywords:** Ischemia, reperfusion, heart, infarct, rabbit
Although the influence of ischemia and reperfusion on cardiac metabolism has been extensively investigated (see (5, 16, 24) for reviews), less well defined are ways in which pharmacological manipulation of cardiac metabolism may be cardioprotective. Under ischemic conditions, myocardial oxidative metabolism is suppressed and glycolysis becomes an important source of ATP generation (32). The increased glycolytic rate in the face of impaired glucose oxidation leads to uncoupling of the two pathways and a buildup of lactate and H⁺ ions (4, 16), a process which may continue during reperfusion (22). This accumulation of protons leads to downstream activation of pathways (Na⁺/H⁺ exchanger, Na⁺/Ca²⁺ exchanger) that result in Ca²⁺ overload, impaired contractile function and/or cell death. Therefore, approaches able to improve glycolytic/oxidative coupling by reducing the glycolytic rate could be expected to be cardioprotective.

One possible approach would be to reduce myocardial glycogenolysis, and thus restrict a source of substrate for glycolysis. Several studies examining the mechanistic basis of ischemic preconditioning have demonstrated in preconditioned hearts that myocardial glycogen stores are depleted, accompanied by attenuated glycogenolysis and glycolysis, and reduced accumulations of lactate and protons (3, 7, 28, 30, 42, 43). Moreover, the loss of myocardial protection in preconditioned hearts correlates with the time course of glycogen recovery (43). Experimental manipulations designed to deplete myocardial glycogen prior to ischemia-reperfusion also have been shown to be cardioprotective (1, 19, 31). Nevertheless, glycogen’s ability to modulate ischemia-reperfusion injury is controversial, in that other studies have failed to show either a link between glycogen depletion and ischemic preconditioning, or a cardioprotective benefit of reducing glycogen stores prior to ischemia and reperfusion (10, 15, 20, 37).
Given that both glycogenolysis (30, 42) and conversion of glycogen phosphorylase to the active form (42) are reduced in preconditioned hearts, and both glycogen phosphorylase activity and glycogenolysis are increased during ischemia in non-preconditioned hearts (8), pharmacological inhibition of glycogen phosphorylase, and thus glycogenolysis, could be postulated to be cardioprotective. A limitation facing past investigations was the lack of pharmacological tools with which to specifically inhibit glycogen phosphorylase, although α-1,6-glucosidase glycogen debranching enzyme inhibitors (miglitol, MOR-14) have been reported to reduce both myocardial glycogen breakdown and infarct size (2, 29). Nevertheless, the putative cardioprotective benefit of inhibiting glycogen phosphorylase has not been formally demonstrated. We recently described a novel class of glycogen phosphorylase inhibitors (12, 27) which bind at a newly discovered allosteric binding site on the enzyme (34). One of these inhibitors is ingliforib (CP-368,296) (13), which inhibits the glycogen phosphorylase isoforms expressed in the myocardium with IC_{50} values of 352 nM (muscle GP) and 150 nM (brain GP), respectively. Thus, to help further clarify the involvement of glycogenolysis in myocardial ischemia-reperfusion injury, we used this novel compound to investigate whether glycogen phosphorylase inhibition is cardioprotective in vitro and in vivo rabbit models of ischemia-reperfusion injury.
MATERIALS AND METHODS

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources (NRC, 1996).

*In vitro (Langendorff) preparation*

Male New Zealand White rabbits (3-4 kg; Covance, Denver, PA) were anesthetized by i.v. administration of sodium pentobarbital (30 mg/kg), followed by intubation and ventilation with 100% O₂ using a positive pressure ventilator. A left thoracotomy was performed, the heart exposed, and a snare (2-0 silk) placed loosely around a prominent branch of the left coronary artery. The heart was rapidly removed from the chest, mounted on a Langendorff apparatus, and maintained by perfusion (non-recirculating) with a modified Krebs solution (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 24.8 mM NaHCO₃, 2.5 mM CaCl₂, and 10 mM glucose) at a constant pressure of 80 mmHg and a temperature of 38.5°C. Perfusate pH was maintained at 7.4 to 7.5 by bubbling with 95% O₂, 5% CO₂. The temperature of the heart was maintained by suspending it in a heated, water-jacketed organ bath. A fluid-filled latex balloon was inserted in the left ventricle and connected by stainless steel tubing to a pressure transducer; the balloon was inflated to provide a systolic pressure of 80 to 120 mmHg, and a diastolic pressure ≤ 10 mmHg. Heart rate (HR), left ventricular systolic and diastolic pressures, and left ventricular developed pressure (LVDP) were recorded using a PO-NE-MAH Data Acquisition and Archive System (Gould Instrument Systems, Valley View, OH). Total coronary flow rate (CF) was determined using an in-line flow probe (Transonic Systems, Inc., Ithaca, NY); CF was
normalized for heart weight. Each heart was allowed to equilibrate for 30 min; if stable left ventricular pressures within the parameters outlined above were not observed, the heart was discarded. Pacing was not used unless the heart rate fell below 180 bpm prior to the 30 min period of regional ischemia; in this case, the heart was paced at 200 bpm, which was the average spontaneous rate observed.

*Langendorff experimental protocols*

Following a 30 min equilibration period, a constant perfusion with ingleforib was initiated, and continued for the duration of the experiment. Thirty minutes after starting the drug perfusion, a 30 min period of regional ischemia was produced by tightening the snare around the branch of the coronary artery. At the end of the ischemic period, the snare was released and the heart reperfused for an additional 120 min. In control hearts, the 30 min of regional ischemia and 120 min of reperfusion was performed in the absence of drug.

*In vivo preparation*

New Zealand White male rabbits (3-4 kg) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.) and a surgical plane of anesthesia was maintained by a continuous infusion of sodium pentobarbital (16 mg/kg/hr) via an ear vein catheter. A tracheotomy was performed through a ventral midline cervical incision and the rabbits were ventilated with 100% oxygen using a positive pressure ventilator. Body temperature was maintained at 38.5°C using a heating pad connected to a YSI temperature controller model 72 (Yellow Springs Instruments, Yellow Springs, MD). Fluid-filled
catheters were placed in the jugular vein for drug administration and in the carotid artery for blood pressure measurements and for blood gas analysis using a model 248 blood gas analyzer (Bayer Diagnostics, Norwood, MA). The ventilator was adjusted as needed to maintain blood pH and pCO$_2$ within normal physiological ranges for rabbits. The heart was exposed through a left thoracotomy at the fourth intercostal space and a 2-0 silk suture was placed around a prominent branch of the left coronary artery. Lead II ECG was measured using an ECG amplifier (Gould Inc, Cleveland, OH) connected to surface ECG electrodes. Arterial pressure was measured using a calibrated strain gauge transducer (Spectromed, Oxnard, CA) connected to the arterial catheter. Heart rate (HR) and mean arterial pressure (MAP) were derived using the PO-NE-MAH Data Acquisition and Archive System. Rate pressure product (RPP) was calculated as the product of HR and MAP. RPP has been previously used as an index of myocardial O$_2$ consumption in this model (17).

In vivo experimental protocols

At least 1 hour after surgery, when arterial pressure, HR and RPP had stabilized for at least 30 min (baseline), rabbits received a bolus of either 15.4 mg/kg of ingliforib or vehicle (administered in 15 sec), followed by a constant infusion of 23.1 mg/kg/hr ingliforib or vehicle at the same dose volume for a total of 3.5 hr. Sixty min after starting the infusion, regional ischemia was produced by tightening the coronary artery snare for 30 min. The snare was released, and the heart was reperfused for an additional 120 min. Myocardial ischemia was confirmed by regional cyanosis and ST segment elevation; reperfusion was confirmed by reactive hyperemia and rapid decline of the ST elevation. At the end of either the ischemic period or reperfusion period, each rabbit was euthanized with an
intravenous overdose of sodium pentobarbital (100 mg/kg). The heart was quickly excised and
prepared for measurement of glycogen phosphorylase (GP) activity and glycogen content, or mounted
on a Langendorff apparatus and perfused with physiological saline at 38.5°C for subsequent
determination of infarct size.

Determination of infarct size

After completion of each experiment (in vitro or in vivo) and with the heart suspended and
perfused on the Langendorff apparatus, the coronary artery snare was re-tightened, and a 0.5%
suspension of fluorescent zinc cadmium sulfide particles (1-10 µm) was perfused through the heart to
delineate the area-at-risk (non-labeled) in the left ventricle (LV) for infarct development. The heart was
removed from the Langendorff apparatus, blotted dry, weighed, wrapped in aluminum foil and stored
overnight at -20°C. Frozen hearts were sliced into 2 mm transverse sections and incubated with 1%
triphenyl tetrazolium chloride in phosphate-buffered saline for 20 min at 37°C to delineate non-infarcted
(stained) from infarcted (non-stained) LV tissue. The infarct area and the area-at-risk were calculated
for each slice of left ventricle using video-captured images and ETC3000 image analysis software
(Engineering Technology Center, Mystic, CT), followed by adding the values for each tissue slice to
obtain the total infarct area and total area-at-risk for each heart. To normalize the infarct area for
differences in the area-at-risk between hearts, the infarct size was expressed as the ratio of infarct area
vs. area-at-risk (% IA/AAR).
Determination of drug concentrations in plasma and protein binding

Quantitation of ingliforib was accomplished by using a LC/MS/MS instrument (API3000, PE-Sciex, Toronto, Canada). An aliquot (10 µl) of plasma, or tissue homogenate (0.2 g/ml in 10 mM sodium phosphate buffer at pH 7.4), was precipitated using 200 µl of methanol-acetonitrile (1:1). Following centrifugation, an aliquot (40 µl) of supernatant was diluted with 200 µl of methanol-acetonitrile (1:1) and the diluted sample (5 µl) injected onto a Phenomenex 40 x 2 mm 5 µm C18 column maintained at 37°C with a run time of ~3 min. The analyte was eluted at 0.5 ml/min flow rate with a linear gradient program consisting of methanol (pump A, 5-95% ramping) and 10 mM ammonium acetate (pump B, 95-5% ramping) produced by two Shimadzu LC-10ADVP binary pumps and a 10 µl static mixer. The column effluent was analyzed using a Turbo Ionspray source at 500°C of a PE-Sciex API-3000 triple quadrupole mass spectrometer. Ingliforib was detected at m/z 456.2 → 193.0 at a retention time of 1.65 min. The calibration curve was prepared by addition of authentic standard (ingliforib) to the control plasma or control tissue homogenate, at concentrations of 0.05 to 50 µg/ml for the plasma and 0.1 to 50 µg/ml for the tissue (6-7 concentrations per standard curve). The standards were processed as the unknowns described above. The standard curve was obtained by fitting linear least squares regression analysis from the peak area of ingliforib with 1/(concentration)$^2$ weighting. The acceptance criterion for the analysis was that all standards used in the curve were ± 20% absolute deviation from the normal value. The absolute tissue-to-plasma concentration ratio was found to be ~1.5 in heart and ~2.7 in liver after a 2 hr infusion (at steady-state).

Plasma protein binding was determined by a 96-well equilibrium dialysis apparatus. Spectra-pro number 2 membranes with molecular weight cutoff of 12-14 kDa were used for the study and were
conditioned for 15 min in deionized water, 15 min in 30% ethanol, and 30 min in sodium phosphate buffer (pH 7.4; 100 mM). Fresh rabbit plasma was obtained from control animals on the day of the study. Plasma samples were spiked with ingliforib to achieve a concentration of 1 µg/ml; 150 µl aliquots (n=6) were loaded into the 96-well equilibrium dialysis apparatus and dialyzed against 150 µl of sodium phosphate buffer. Equilibrium was achieved by incubating the 96-well equilibrium dialysis apparatus in a 37°C shaking water bath at 155 rpm for 5 hr. At the end of the dialysis period, 10 µl of the dialyzed plasma and 90 µl of the buffer were transferred to HPLC vials containing 100 µl of methanol-acetonitrile (1:1). Control buffer (90 µl) was added to the vial containing the dialyzed plasma sample, and 10 µl of control plasma was added to the vial containing the buffer sample. The vials were vortexed, centrifuged, and the supernatant assayed by the LC/MS/MS assay described above. The plasma unbound fraction (fu) was estimated by the ratio of drug concentration in the buffer sample to the drug concentration in the plasma sample. (The mean fu for ingliforib was 0.036 ± 0.002 in rabbit plasma).

**Determination of plasma glucose and lactate concentrations**

Blood samples were collected in heparinized tubes, followed by centrifugation and collection of the plasma. Plasma glucose and lactate concentrations were determined using a Roche/Hitachi 912 Clinical Autoanalyzer (Roche Diagnostics Corporation, Indianapolis, IN) using the Glucose HK and Lactate reagent systems (Roche Diagnostics), respectively.
Measurement of myocardial glycogen phosphorylase activity

Hearts were rapidly removed from the animals at the end of the 30 min ischemic period and perfused with ice-cold saline. The ischemic myocardium was identified as the region not cleared of blood by the saline perfusion and was dissected free of the remainder of the heart. In hearts in which infarct size was determined, the right ventricular free wall was used for determining glycogen phosphorylase activity. Myocardial samples were frozen in liquid nitrogen, and stored at –80°C until analysis. Heart samples (75 mg) were homogenized at a 1:39 dilution in 50 mM MES, 100 mM KF, 5 mM EDTA, 0.4% BME, pH 6.1 using a Vertis Handishear at 30,000 rpm for 15 sec. Samples were then centrifuged at 3000 X g for 5 min and the supernatant transferred for analysis of glycogen phosphorylase activity by modification of the method of Gilboe et al. (9). In brief, 60 µl reagent mix (50 mM MES, 75 mM KF, 0.8% glycogen, 50 mM glucose-1-phosphate, 45 nCi [14C]-glucose-1-phosphate (Perkin Elmer, NEC390), +/- 3.3 mM AMP, pH 6.1) was pipetted into 12x75 glass tubes. To initiate the reaction, a 30 µl sample was added to the tubes in duplicate, and the reaction allowed to proceed at 37°C for 30 min. The reaction was terminated by removing a 75 µl aliquot and spotting onto a 15x15 mm Whatman 31ET CHR filter paper. Filters were washed 3 times with 60% ethanol, dried with acetone, and placed in 7 ml scintillation vials with 5.5 ml scintillation fluid (Beckman Coulter Ready Safe), and counted on a Wallac 1409 Liquid Scintillation Counter. The results are expressed in units of dpm/mg right ventricle, analyzed in duplicate. Cardiac glycogen phosphorylase a (GPa) activity is defined as the measured activity in the absence of AMP (-AMP); total GP activity (GPa+Gpb) is defined as the measured activity in the presence of AMP (+AMP).
Measurement of myocardial glycogen content

At the end of the 30 min period of regional ischemia, hearts were removed from the animals and rapidly perfused with ice-cold saline. The ischemic myocardium was identified as the region not cleared of blood by the saline perfusion and was dissected free of the remainder of the heart. Myocardial samples were frozen in liquid nitrogen, and stored at −80°C until analysis. Approximately 25 mg of frozen tissue was added to 16x100 mm glass test tubes, followed by addition of 1.5 ml of 30% KOH. The tubes were heated in a 60°C oven for 30 min and repeatedly agitated. Two ml of 100% ethanol and 250 µl of saturated sodium sulfate were added to each sample. The tubes were heated for 3 min at 90°C, and then placed on ice for 15 min. Samples were centrifuged at 4°C, 3200 x g for 5 min. After aspiration of the supernatants, the pellets were dried for 60 min in a 60°C oven. The pellets were then hydrolyzed in 1 ml of 5N HCl for 1 hr in a 60°C oven. Samples were cooled at room temperature and neutralized with 1 ml of 5N NaOH and 3 ml of deionized water. For each sample, 2 ml of anthrone reagent (200 mg of anthrone (Sigma) in 100 ml H₂SO₄) was added to a 16x100 mm test tube, followed by addition of 1 ml of neutralized heart hydrolysate or 1 ml of glucose standard. Tubes were vortexed and heated at 90°C for 15 min; samples were then immediately cooled at 4°C. Two hundred µl was transferred in duplicate to a 96 well plate, which was read at 620 nm in a SpectroMax Plus microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

Data expression and analysis

Data are expressed as the mean ± SE. Between group comparisons of in vitro and in vivo areas-at-risk expressed as a percent of left ventricular areas (% AAR/LV) were compared using ANOVA. Temporal comparisons of in vivo hemodynamic parameters, plasma glucose concentrations,
and plasma lactate concentrations between ingliforib and vehicle control were performed using ANOVA with repeated measures. In vitro hemodynamic, glycogen content, and glycogen phosphorylase activity comparisons were performed by t-test, while in vitro and in vivo % IA/AAR values were compared using a Mann-Whitney test; a Bonferroni correction was applied to multiple comparisons. A P value of less than 0.05 was considered statistically significant.

*Drugs and drug preparation*

The synthesis of ingliforib (1H-Indole-2-carboxamide, 5-chloro-N-[(1S,2R)-3-[(3R,4S)-3,4-dihydroxy-1-pyrrolidinyl]-2-hydroxy-3-oxo-1-(phenylmethyl)propyl]- (9CI) ) has been reported (13) and was performed at Pfizer Global Research and Development (Groton, CT). Drug administered to the isolated hearts was dissolved in DMSO and diluted in buffer; the final DMSO concentration was less than 0.1%, which had no effect on infarct size (39). For the in vivo studies, ingliforib was dissolved in 25% sulfobutylether 7-β cyclodextrin sodium (CAPTISOL, Cydex, Inc., Overland Park, KS) in 0.01 M phosphate-buffered saline at a concentration of 13 mg/ml.
RESULTS

In the isolated rabbit hearts, baseline HR, CF and LVDP values for each of the treatment groups were similar prior to the regional ischemia and are shown in Table 1. LVDP and CF were significantly (P < 0.05) reduced in all groups by occlusion of the coronary artery, confirming that ischemia was achieved in all groups. In anesthetized rabbits, baseline HR, MAP and RPP were similar between vehicle control and ingliforib -treated groups (Fig. 2). Administration of ingliforib did not significantly affect HR, LVDP or CF in the isolated hearts (Table 1), nor did this compound affect MAP or RPP in vivo (Fig. 2B and 2C). A modest reduction in HR of the ingliforib group vs. the vehicle control group (P < 0.05) was observed during the reperfusion period in the in vivo studies (Fig 2A).

Ingliforib elicited a concentration-dependent reduction in infarct size in the isolated rabbit hearts (Fig. 3). The maximum reduction in infarct size achieved with 10 µM ingliforib was 69% (control: 52 ± 2% IA/AAR; 10 µM ingliforib: 16 ± 2% IA/AAR, P < 0.05). Area-at-risk expressed as a percent of left ventricular area (% AAR/LV) for the ingliforib treatment groups did not differ significantly (P ≥ 0.05) from that of the control group (33 ± 2%). In anesthetized rabbits, a dose of ingliforib was selected to achieve free drug plasma concentrations comparable to an EC_{50} concentration (1.2 µM, 0.55 µg/ml) estimated from the isolated heart experiments. This dose of ingliforib (15 mg/kg loading dose; 23 mg/kg/hr infusion) provided a plasma concentration of 21.0 ± 1.4 µg/ml just prior to the regional ischemia; ingliforib is 96.5% protein-bound, yielding a free drug plasma concentration of 0.7 µg/ml (1.5 µM). At this dose, infarct size was significantly reduced by 51% in vivo (Fig. 4) (vehicle control: 65 ± 3% IA/AAR; ingliforib: 31 ± 4% IA/AAR, P < 0.05); the % AAR/LV did not differ (P > 0.05) between these groups (control: 41 ± 5%; ingliforib: 42 ± 4%).
Glycogen phosphorylase activity was significantly \((P < 0.05)\) inhibited in the myocardium from the ingliforib-treated animals (Fig. 5). At the end of the 30 min period of regional ischemia, \(\text{GP}_{a}\) and total GP activity were reduced by 65% and 40%, respectively, in the ischemic myocardium, and 41% and 33%, respectively, in the non-ischemic myocardium (Fig. 5). In addition, the ingliforib-dependent \(\text{GP}_{a}\) inhibition was significantly \((P < 0.05)\) greater in the ischemic vs. non-ischemic myocardium. Glycogen phosphorylase \(a\) and total GP activity were similar in the ischemic and non-ischemic myocardium from the vehicle-treated animals (Fig. 5). Inhibition of GP activity by ingliforib was also verified in hearts in which infarct size was determined, by measuring GP activity in the right ventricle; \(\text{GP}_{a}\) and total GP activity were reduced by 83% (vehicle: \(4164 \pm 699\) dpm/mg tissue, ingliforib: \(666 \pm 115\) dpm/mg tissue; \(n = 8\)) and 63% (vehicle: \(7044 \pm 1003\) dpm/mg tissue, ingliforib: \(2622 \pm 247\); \(n = 8\)), respectively, at the end of the reperfusion period.

To establish inhibition of glycogenolysis by ingliforib, glycogen content in the ischemic and non-ischemic myocardium from vehicle- and ingliforib-treated anesthetized rabbits was measured at the end of the 30 min period of regional ischemia. Myocardial glycogen stores were significantly \((P < 0.05)\) reduced in the ischemic vs. non-ischemic myocardium, whereas ingliforib treatment significantly \((P < 0.05)\) preserved glycogen content in the ischemic myocardium (Fig. 6).

Systemic GP inhibition by the cardioprotective dose of ingliforib was assessed by measuring plasma glucose and lactate concentrations. Baseline plasma glucose and lactate concentrations were comparable in vehicle and ingliforib-treated groups (Fig. 7). In vehicle control animals, a rise in plasma glucose and lactate concentrations were observed during the ischemic period, which peaked at the end of the ischemia and remained elevated during the subsequent reperfusion. Ingliforib significantly \((P < 0.05)\) blunted the rise in both glucose and lactate plasma concentrations (Fig. 7).
DISCUSSION

Under the anaerobic conditions of myocardial ischemia, the heart relies primarily on glycolysis for ATP generation (32). Myocardial glycogen and glycogenolysis are important sources of glycolytic substrate, particularly when coronary flow is limited (5) and exogenous glucose delivery to the heart is reduced. However, because oxidation is impaired during ischemia, glycolysis and oxidation become uncoupled, leading to a build-up of lactate and H⁺ ions (4, 16). This deleterious process can continue during reperfusion, as glycolysis, and fatty acid oxidation (upon which the heart primarily depends for its energy demands), recover quickly and may exceed pre-ischemic rates (21, 25, 36). Consequently, glucose oxidation remains markedly depressed, glycolytic/oxidative uncoupling continues, and the myocardial lactate/proton load persists (24, 25, 35). Considering these observations, it follows that inhibition of glycogenolysis would be cardioprotective due to a reduction in glycolytic substrate and improved glycolytic/oxidative coupling; indeed, studies in preconditioned hearts have demonstrated glycogenolysis is significantly attenuated (30, 42). Weiss et al. established this decrease in glycogenolysis (likely due to reduced conversion of glycogen phosphorylase to the α or “active” form during early ischemia) resulted in a diminished glycolytic rate and decreased accumulation of lactate and H⁺ ions (42). Conversely, glycogen phosphorylase activity and glycogenolysis have been reported to increase markedly during global low-flow ischemia in non-preconditioned hearts (8).

To formally establish that pharmacological inhibition of glycogen phosphorylase is cardioprotective, we used a novel glycogen phosphorylase inhibitor, ingliforib, in well-established models of myocardial ischemia-reperfusion injury. Ingliforib inhibits the myocardial glycogen phosphorylase isoforms (muscle and brain) with IC₅₀s of 352 nM and 150 nM, respectively, and is also
a potent inhibitor of the liver isoform (IC$_{50}$ of 52 nM). Using this compound, we have demonstrated for
the first time that inhibition of myocardial glycogen phosphorylase provides significant protection from
myocardial ischemia-reperfusion injury. The cardioprotection afforded by ingliforib in the isolated rabbit
heart was concentration-dependent; 10 µM ingliforib reduced infarct size by 69%, which is similar to
the efficacy of other cardioprotective agents we have characterized in this model (e.g., adenosine A$_3$
receptor agonists, NHE inhibitors, NCX inhibitors, aldose reductase inhibitors) (18, 26, 40, 41). In
vivo studies, which were designed to target a free plasma concentration equivalent to the EC$_{50}$ we
estimated from the isolated heart studies, resulted in a 50% reduction in infarct size. In addition,
glycogen phosphorylase inhibition was confirmed in vivo, both within the heart and systemically.
Glycogen phosphorylase activity (total and GP$_{a}$) was significantly blunted by ingliforib in the ischemic
and non-ischemic myocardium, and glycogen stores preserved in the ischemic myocardium.
Systemically, plasma glucose and lactate concentrations were significantly lowered by ingliforib
treatment. However, ingliforib’s in vivo cardioprotective efficacy was independent of systemic glycogen
phosphorylase inhibition because ingliforib reduced infarct size in vitro, and equivalent drug exposure in
vitro and in vivo produced similar reductions in infarct size. It was noteworthy that neither in the isolated
heart, nor in vivo, were any significant unwanted cardiovascular effects observed, i.e., changes in
cardiac function, coronary flow, or mean arterial blood pressure. In vivo, heart rate was minimally
reduced in the ingliforib-treated group; while this could be viewed as a trend towards reducing
myocardial oxygen consumption, a significant drop in RPP was not observed. Our results show that
partial (65-83%) inhibition of cardiac glycogen phosphorylase was associated with reduced infarct area
in the absence of other untoward effects on cardiac function. Whether complete inhibition of cardiac
glycogen phosphorylase in the ischemic myocardium would produce a similar profile, or would lead to untoward effects due to energy substrate deprivation, remains to be determined.

Our data support earlier studies in which \( \alpha \)-1,6-glucosidase glycogen debranching enzyme inhibitors (miglitol, MOR-14) preserved myocardial glycogen content, attenuated lactate accumulation and reduced infarct size (2, 29). The demonstration that ingliforib has similar effects on myocardial glycogen content and infarct size further underscores the significance of inhibiting glycogenolysis for ameliorating myocardial ischemia-reperfusion injury, while validating glycogen phosphorylase as a cardioprotective molecular target. Moreover, the in vivo efficacy of ingliforib and lack of adverse effects suggests that glycogen phosphorylase inhibition may be a viable therapeutic approach for achieving clinical cardioprotection. As a pharmacological tool, ingliforib should facilitate further study of the role of glycogen phosphorylase in the physiology/pathophysiology of the heart and other organs.

Although the current studies focused on the response of normal hearts to ischemia-reperfusion injury, glycogen phosphorylase inhibitors are being investigated for the treatment of diabetes (ingliforib reduced plasma glucose and lactate in our normal rabbits, and reduces plasma glucose in diabetic models (Hoover et al., in preparation)). Moreover, the diabetic patient population is at increased risk for developing cardiovascular complications, including myocardial infarction (11, 14). Although controversial (6, 33), the almost complete reliance of the diabetic heart on fatty acid metabolism and minimal glucose oxidation rate may increase the sensitivity to ischemic injury due to the considerable uncoupling of glycolysis and glucose oxidation (23, 24, 33, 38). Thus, one could speculate that a glycogen phosphorylase inhibitor might not only treat diabetes per se, but may also protect the diabetic heart already predisposed to ischemic injury. Future studies to address this possibility should be considered.
In conclusion, we have demonstrated that a novel glycogen phosphorylase inhibitor, ingliforib, inhibits myocardial glycogen phosphorylase, preserves glycogen stores, and provides significant cardioprotection from ischemia-reperfusion injury. The benefit resulting from glycogen phosphorylase inhibition may ultimately be due to a reduction in myocardial glycolysis, an improvement in glycolytic/oxidative coupling, and a reduction in intracellular proton load. Moreover, the cardioprotection is achieved without eliciting undesirable changes in cardiac function or hemodynamics. Thus, glycogen phosphorylase inhibition may represent an attractive target for clinical cardioprotection, and for treating diabetic patients at increased risk for cardiovascular complications.
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REFERENCES


42. Weiss RG, de Albuquerque CP, Vandegaer K, Chacko VP and Gerstenblith G. Attenuated glycogenolysis reduces glycolytic catabolite accumulation during ischemia in preconditioned rat hearts. 

43. Wolfe CL, Sievers RE, Visseren FLJ and Donnelly TJ. Loss of myocardial protection after preconditioning correlates with the time course of glycogen recovery within the preconditioned segment. 
Figure Legends

Fig. 1. Structure of ingliforib (CP-368,296).

Fig. 2. Effect of ingliforib and vehicle control on HR (A), MAP (B) and RPP (C) in anesthetized rabbits. Ingliforib or vehicle was administered by constant infusion beginning 60 min prior to the regional ischemia, and continued for the duration of the experiment, as described under Materials and Methods. Ingliforib had no significant effect on MAP or RPP, although a modest (P < 0.05) decrease in HR vs. vehicle control was observed during the reperfusion period. Data are the mean ± SE for each group, n = 8. *Significantly different (P < 0.05) from vehicle.

Fig. 3. Effect of ingliforib on % IA/AAR in isolated rabbit hearts. Ingliforib was constantly perfused through the hearts beginning 30 min prior to the regional ischemia and continued for the duration of the experiment, as described in Materials and Methods. Infarct areas and area-at-risk were determined by image analysis and infarct area was normalized for area-at-risk (% IA/AAR). Data from each heart are presented, along with the mean ± SE for each group, n = 6 - 10. *Significantly different (P < 0.05) from control.
**Fig. 4.** Effect of in vivo administration of ingliforib on % IA/AAR in anesthetized rabbits. Ingliforib or vehicle was administered by constant infusion beginning 60 min prior to the regional ischemia, and continued for the duration of the experiment, as described under *Materials and Methods*. Infarct area and area-at-risk were determined by image analysis and infarct area was normalized for area-at-risk (% IA/AAR). Data from each heart are presented, along with the mean ± SE for each group, n = 8.

* Significantly different (P < 0.05) from vehicle.

**Fig. 5.** Effect of ingliforib on myocardial glycogen phosphorylase α (GPα) and total glycogen phosphorylase (GP) activity at the end of 30 min of regional ischemia. Ingliforib or vehicle was administered by constant infusion to anesthetized rabbits, beginning 60 min prior to the regional ischemia. The ischemic and non-ischemic myocardium was harvested and glycogen phosphorylase activity determined as described under *Materials and Methods*. Data are the mean ± SE for each group, n = 7. * Significantly different (P < 0.05).

**Fig. 6.** Effect of ingliforib on myocardial glycogen stores after 30 min of regional ischemia. Ingliforib or vehicle was administered by constant infusion to anesthetized rabbits, beginning 60 min prior to the regional ischemia. The ischemic and non-ischemic myocardium was harvested and glycogen content determined as described under *Materials and Methods*. Data are the mean ± SE for each group, n = 7. * Significantly different (P < 0.05); NSD: not significantly different (P ≥ 0.05).
**Fig. 7.** Effect of ingliforib and vehicle control on plasma glucose (A) and plasma lactate (B) in anesthetized rabbits. Ingliforib or vehicle was administered by constant infusion beginning 60 min prior to the regional ischemia, and continued for the duration of the experiment, as described under *Materials and Methods*. Blood samples were obtained at the indicated time points and glucose and lactate concentrations determined. Data are the mean ± SE for each group, n = 8. *Significantly different (P < 0.05) from vehicle.*
Table 1. Cardiac function and coronary flow data from isolated rabbit hearts

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pre-ischemia</th>
<th></th>
<th>End ischemia</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR (beats·min⁻¹)</td>
<td>CF (ml·min⁻¹·g⁻¹)</td>
<td>LVDP (mmHg)</td>
<td>HR (beats·min⁻¹)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>213 ± 10</td>
<td>7.1 ± 0.4</td>
<td>101 ± 2</td>
<td>203 ± 11</td>
</tr>
<tr>
<td>Ingliforib, 0.1 µM</td>
<td>6</td>
<td>208 ± 11</td>
<td>6.5 ± 0.2</td>
<td>99 ± 4</td>
<td>199 ± 9</td>
</tr>
<tr>
<td>Ingliforib, 1 µM</td>
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<td>207 ± 7</td>
<td>7.2 ± 0.3</td>
<td>95 ± 3</td>
<td>194 ± 6</td>
</tr>
<tr>
<td>Ingliforib, 10 µM</td>
<td>10</td>
<td>203 ± 3</td>
<td>7.0 ± 0.3</td>
<td>93 ± 3</td>
<td>200 ± 5</td>
</tr>
</tbody>
</table>

HR: heart rate; CF: total coronary flow; LVDP: left ventricular developed pressure; *P < 0.05 vs. pre-occlusion values; mean ± SE.
Inliforib (CP-368,296)

Figure 1
Figure 2A
Figure 2B
Figure 2C
Figure 3
Figure 4
Figure 5
Figure 6

Myocardial Glycogen Content (mg/g wet wt)

- **Vehicle**
- **Ingliforib**

Ischemic Area

Non-Ischemic Area

*NSD*

* *
Figure 7A

Figure 7B